Expression of PGP 9.5 by Enteric Neurons in Horses and Donkeys with and without Intestinal Disease

SPONTANEOUSLY ARISING DISEASE

Short Title: Expression of PGP 9.5 by Equine Enteric Neurons

Expression of PGP 9.5 by Enteric Neurons in Horses and Donkeys with and without Intestinal Disease

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Summary

Intestinal motility disorders are an important problem in horses and donkeys and this study was carried out in order to evaluate the enteric neurons in animals with and without intestinal disease. Surplus intestinal tissue samples were collected from 28 horses undergoing exploratory laparotomy for colic. In addition, surplus intestinal samples from 17 control horses were collected immediately following humane destruction for clinical conditions not relating to the intestinal tract. Similar samples were also collected during routine post-mortem examinations from 12 aged donkeys; six animals were humanely destroyed for conditions related to the intestinal tract, while the remaining six were humanely destroyed for other reasons including dental and orthopaedic diseases. Tissue samples were fixed in formalin and immunohistochemical labelling was performed targeting the enteric neurons using a polyclonal antibody specific for the neuronal marker PGP 9.5. The distribution and density of neuronal networks were assessed qualitatively and semi-quantitatively. There was strong PGP 9.5 expression in both the horse and donkey samples and labelling was detected throughout the tissue sections. In both species, PGP 9.5-immunoreactive nerve fibres were detected in all layers of the intestinal tract, both in large and small intestinal samples. Networks of enteric neurons were present in the donkey with a similar distribution to that seen in the horse. There was no demonstrable difference in enteric neuronal density and distribution in the groups of animals with intestinal disease compared with those without, apart from two (out of 28) horses with intestinal disease that showed a marked reduction in PGP 9.5 immunoreactivity. Apart from these two animals, this total cohort analysis differs from some previously observed findings in horses with intestinal disease and may therefore reflect the different pathophysiological processes occurring in varying intestinal conditions resulting in colic both in the donkey and horse.

Keywords: donkey; horse; neuron; colic

Introduction

Disorders of the gastrointestinal tract are among the most common problems in equine medicine. Colic (abdominal pain) is reported by insurance companies and universities as the single greatest killer of horses (White, 1990). The incidence of colic in the horse has been reported as four to 10 cases per 100 horses per year (Tinker *et al.*, 1997; Traub-Dargatz *et al*., 2001; Archer and Proudman, 2006; Dukti and White, 2009). The economic impact on the equine industry is significant with an annual estimated cost in the USA of over $115 million (Traub-Dargatz *et al*., 2001).

Colic disorders also represent a significant problem in donkeys and impaction of the pelvic flexure of the large colon has been reported as the most common cause of colic in the UK donkey population (Cox *et al.*, 2007). That study also identified risk factors for developing impactions, which included old age and dental disease, as well as previous colic episodes (Cox *et al.*, 2007). Du Toit *et al.* (2008) further identified the presence of cheek teeth diastemata as a factor associated significantly with colic-related illness in the donkey.

The precise cause of gastrointestinal dysfunction is often unknown in cases of colic (Archer and Proudman, 2006). These syndromes may be linked to alterations in the motility control systems, namely the enteric nervous system, the interstitial cells of Cajal (ICC) and the intestinal smooth muscle. Horses with chronic obstructive disorders of the large colon or recurrent caecal impactions have been shown to have reduced densities of neurons in the large colon or caecum (Schusser and White, 1997; Schusser *et al.*, 2000). Additionally, a reduction in ICC density has been demonstrated in the pelvic flexure of horses with large intestinal obstructive disorders, suggesting a possible involvement of ICC in equine intestinal dysmotility (Fintl *et al.*, 2004). Recently, a reduction in enteric neuronal activity and ICC

immunoreactivity in cases of colic in horses has been reported (Pavone *et al*., 2012). Finally, in donkeys, a recent study showed there was no difference in ICC density and distribution in animals with and without intestinal disease (Fintl *et al*., 2010a), but to the authors' knowledge, investigation of the neuronal component of intestinal motility has not been evaluated previously in the clinical setting in this species.

The aim of the present study was to compare the enteric neuronal profile in normal (free from intestinal disease) versus diseased animals (both horses and donkeys), using a neuronal immunohistochemical marker, protein gene product (PGP) 9.5.

Materials and Methods

Samples

Intestinal samples were collected from all animals at anatomically-defined sites. These were: the ileum (at a position level with the midpoint of the ileocaecal fold) and the apex of the pelvic flexure (located at the junction between the left ventral colon and left dorsal colon). In some horse surgical cases, jejunum was collected from the non-ischaemic margin of the resected small intestine. The differing numbers and age ranges of horse and donkey samples detailed below reflects the access to material from the two species in these clinical caseload settings.

Horses. Intestinal samples from 17 control horses were collected immediately (within 1 h) after humane destruction for clinical conditions not relating to the intestinal tract. The ages of the animals in this group ranged from 1–33 years (median 11.0 years, mean 12.3 years) and these animals comprised of one stallion, 11 geldings and five mares. The samples collected included a section of ileum and/or pelvic flexure as described. It was not always possible to obtain both ileal and pelvic flexure samples from all horses included in this study, so a total of

12 ileal and eight pelvic flexure control samples were collected (Table 1). The small intestinal control group ranged in age from 1–33 years (median 11.5 years, mean 13.5 years) and comprised of one stallion, eight geldings and three mares, while the large intestinal control group comprised of one stallion, five geldings and two mares with an age range of 1–19 years (median 6.5 years, mean 8.0 years).

In addition, intestinal tissue samples were collected from 28 horses undergoing exploratory laparotomy for the evaluation and treatment of colic (Table 1). The age range of this group was 2–24 years (median 11.0 years, mean 11.0 years) and it comprised of 14 geldings and 14 mares. Small intestinal tissue sections were obtained from the non-ischaemic margin of resected small intestinal tissue from nine horses with an obstructive lesion of this anatomical region. The samples collected included jejunum (n = 4) or ileum (n = 5). Every effort was made to collect samples that were similar or as close to the anatomical site of the control animals as possible. The age range of this group was 7–24 years (median 14.0 years, mean 14.8 years) and it consisted of six geldings and three mares. In addition, samples of pelvic flexure collected from 19 horses with a large colon obstructive disorder were evaluated. The samples evaluated were all surplus tissue following a pelvic flexure apex biopsy submitted for histological analysis and collected during an enterotomy procedure prior to large colon evacuation. The age range of this group was 2–24 years (median 9.0 years, mean 9.3 years) and it consisted of eight geldings and 11 mares.

The animals in this study included material from 39 horses from previous immunohistochemical and molecular studies of ICC (Fintl *et al.*, 2004, 2010b). Samples from six additional horses were included. The reason for including horses from previous studies was to obtain a higher collective sample number, as there was not always enough tissue available to be used in all immunohistochemical studies. This was particularly true for the large colon colic samples, which were all surplus material following a pelvic flexure apex

biopsy. In addition, this current study evaluated a different component of the intestinal motility control system, namely the enteric neurons. Samples were from horses admitted to the equine hospitals at the Universities of Edinburgh and Liverpool and were collected with the informed, written consent of the owners.

Donkeys. Intestinal tissue samples were collected from 12 donkeys following routine post-mortem examinations. In seven animals, humane destruction was related to clinical signs indicating an abdominal problem, while in the remaining five donkeys, other conditions were diagnosed (Table 2). All animals were subsequently grouped into either the control group (i.e. animals that following post-mortem examination were determined not to have intestinal disease) or into the disease group (animals with an intestinal disorder). All donkeys were aged 26–35 years (median 28 years, mean 29.5 years) and the population consisted of one stallion, seven geldings and four mares. The age range of the control group was 26–32 years (median 27 years, mean 28.1 years) and this group comprised of two geldings and four mares. The age range for the colic group was 27–35 years (median 27.5 years, mean 30.3 years) and it comprised of one stallion and five geldings. One animal (donkey 6) was humanely destroyed on the basis of displaying clinical signs of colic, but was subsequently diagnosed *post mortem* to have a chronic liver problem (Table 2). As a consequence, this animal was included in the control group rather than in the intestinal disease group. All animals with a suspected abdominal problem had been treated medically (including with oral laxatives, oral or intravenous fluid therapy and analgesic drugs). However, as these treatments failed to resolve the problem, the decision was taken to humanely destroy the individual animals rather than to pursue further treatment such as exploratory abdominal surgery. The latter was not considered to be in the animals' best interests, primarily due to their advanced age and general physical condition, which rendered them poor surgical candidates.

A segment of ileum (level with the midpoint of the ileocaecal fold) and a sample of the pelvic flexure (from the junction of the left ventral colon and left dorsal colon) were collected and then analyzed from all animals apart from donkeys 9 and 10 where only a section of pelvic flexure was analyzed and donkey 4 where only ileum was analyzed. As with the horse studies, 12 of the samples were collected from animals used in a previous immunohistochemical study of ICC (Fintl *et al.*, 2010a), which, as mentioned above, evaluated a different component of the intestinal motility control system. The post-mortem examination performed in all animals included assessment of their teeth (Fintl *et al.*, 2010a). All samples were collected with the permission of, and in collaboration with, The Donkey Sanctuary, Sidmouth, Devon, which owned all of the donkeys included in the study.

Histology and Immunohistochemistry

Samples were fixed in 10% phosphate buffered formalin for at least 24 h. Tissue processing consisted of rinsing the tissue samples in running tap water for 1 h prior to placing them in graded sucrose solutions (10% and 30% sucrose in phosphate buffered saline) in order to cryoprotect the samples. The samples were frozen rapidly in isopentane (BDH Laboratory Supplies, Poole, UK) pre-cooled in liquid nitrogen or dry ice and subsequently sectioned (10 μm). All sections were mounted on Tespa-coated (3-aminopropyltriethoxysilane; Sigma Aldrich, Poole, UK) slides and allowed to air dry overnight. After washing the sections in phosphate buffered saline (PBS), they were incubated for 30 min in H_2O_2 0.3% in methanol in order to quench endogenous peroxidase activity. The sections were then incubated for 1 h in 1% goat serum (Vector Laboratories, Burlingame, California, USA) in order to block non-specific antibody binding. After subsequent washes with PBS, the sections were incubated overnight at 4°C in a humid chamber with rabbit polyclonal antiserum specific for PGP 9.5 (UltraClone, Wellow, Isle of Wight, UK), at a concentration of 1 in 8,000. The tissue sections

were then washed with PBS prior to a 1 h incubation with biotin-conjugated goat anti-rabbit immunoglobulin (Vector Laboratories) at a concentration of 1 in 200. Labelling was 'visualized' using the avidin–biotin method (ABC; Vectastain Elite ABC Kit, Vector Laboratories) with a 3, 3' diaminobenzidine substrate (DAB; BDH Laboratory Supplies). Sections were dehydrated in ethanol, cleared in xylene and then mounted under DPX (Merck, Glasgow, UK).

For all batches processed, both negative and positive controls were included. In negative controls, the primary antibody was replaced with normal rabbit serum. This resulted in the complete absence of immunolabelling. Positive controls were based on the use of tissue from normal control animals in which abundant immunoreactivity for PGP 9.5 had been demonstrated previously.

All samples were evaluated in blinded fashion on two occasions (1 week apart) by one observer (CF) similar to the technique described by Prince *et al.* (2003). A semiquantitative grading system, as described for previous equine ICC studies (Hudson *et al*., 2001; Fintl *et al*., 2004), was used and modified for this study. The grades given were based on the scrutiny of four adjacent 10 μm tissue sections from each sample from each animal. PGP 9.5 immunoreactivity was graded as absent (grade 0), sparse (grade 1), moderate (grade 2) or abundant (grade 3). Immunoreactivity of the myenteric plexus region and circular muscle was evaluated separately. Where mucosa was included in the sections, immunoreactivity of this region was also evaluated; this involved assessment mucosal nerve (including fibre) density. Depending on the size and thickness of the original tissue section, it was not always possible to obtain complete full-thickness samples in all animals for this study. A Mann–Whitney test assuming a significance level of 5% was used when analyzing the data. Variability between the grades given on the two separate occasions was also assessed using the same statistical method.

In addition to immunohistochemical labelling, all processed tissues were stained with haematoxylin and eosin (HE) in order to assess tissue integrity. This was performed to help clarify whether reduced or absent immunoreactivity was a primary finding or secondary to a degree of tissue autolysis. Only samples with good tissue integrity (and not worse than a mild degree of mucosal degradation) were included in order to ensure that immunohistochemical grading was reliable. As no significant degree of tissue autolysis was present in any of the samples collected, all were included in the study.

Results

There was strong expression of PGP 9.5 in the horse and donkey samples and this was detected throughout the tissue sections. In the horse, PGP 9.5-immunoreactive nerve fibres were detected in all layers of the intestinal tract, both in large and small intestinal samples. Ganglia were observed in the submucosal and myenteric plexuses. In the mucosa, lamina propria and muscularis mucosa the enteric neuronal axons were clearly evident, forming a dense network and, in the ileal samples, these extended into the mucosal villi (Fig. 1). Immunoreactive axons were also observed in the submucosa as well as the circular and longitudinal smooth muscle layers; with the nerve fibres generally having an orientation that was parallel to the muscle fibres and uniformly distributed throughout the muscle layers (Figs. 2 and 3). Ganglia were observed both in the submucosal and myenteric plexus regions. The tissue sections were cut in a plane parallel to the orientation of the circular muscle fibres, with the longitudinal muscle layer presenting in transverse section. However, it was sometimes difficult to obtain true parallel sections, especially in the smaller pelvic flexure biopsy samples. Therefore, in a number of slides the nerve fibres would have been transected, thereby showing a punctuate positivity rather than a parallel linear positive reaction. Despite this, it was still possible to estimate the degree of immunoreactivity due to the strong

immunolabelling this antibody provides. The size and number of ganglionic collections of neurons varied, with the size being clearly dependent on the angle of section. However, the ganglia observed in the myenteric plexus were larger and more numerous than those observed in the submucosal region. Again, because of the angle of sectioning, it was not possible to reliably count the individual cell bodies within the ganglia. Because only a limited amount of tissue was available and this did not always include mucosa, the PGP 9.5 density in this region could not be assessed in all cases. Owing to the clinical nature of the study (i.e. collection of clinically-derived tissues), it was considered reasonable to assess the samples without mucosa as being viable, since none of the samples collected in a similar fashion that included mucosa showed significant signs of autolysis. In addition, these samples showed no preservation-related abnormalities in the other intestinal layers.

In the donkeys, similar patterns of PGP 9.5 immunoreactivity were observed throughout the intestinal layers, both in large and small intestinal samples. In the myenteric plexus region there was also an immunoreactive band of axons between the ganglia (Fig. 4). This differed from that observed in the submucosa where only individual ganglia were observed (Fig. 5). A subtle subjective difference between donkey and horse PGP 9.5 immunoreactivity was that the donkey nerve networks (fibres and ganglia) appeared more delicate in nature. Both in horses and donkeys, there was evidence of strong PGP 9.5 immunoreactivity even with increasing age of the animals.

Comparison was made between normal and diseased animals with respect to the PGP 9.5-immunoreative neuronal profiles. In the horse study, the PGP 9.5 density was assessed and compared in the mucosa, circular muscle layer and the myenteric plexus of both the ileum and pelvic flexure in normal and diseased animals. The *P* values for these analyses are shown in Table 3.

As seen from these data, there was no significant difference in the PGP 9.5 density in any of the anatomical areas in either of the groups. There were no animals with absent immunoreactivity, although two animals with a large colon obstructive disorder had markedly reduced immunoreactivity throughout the pelvic flexure tissue sample, which was in stark contrast to the majority of normal and diseased horses. There was no significant difference in the grades given by the observer on the two separate occasions ($P = 0.80$) indicating good grading consistency.

Similar observations were made in the donkey groups (Table 4). No significant difference was observed in the density of PGP 9.5 immunoreactivity in the myenteric plexus region between the small intestinal samples collected from diseased animals and those of the controls. Similarly, there was no significant difference in the density of PGP 9.5 immunoreactivity observed in the circular muscle layer or the mucosa between these two groups. Equally, in the donkey large intestine, there was no significant difference in the density of PGP 9.5 immunoreactivity in the myenteric plexus region or the circular muscle layer between control and diseased animals in the pelvic flexure samples. Similarly, there was no significant difference in the density of PGP 9.5 immunoreactivity of the mucosa between these two groups. It is worth re-emphasising here that although small intestinal samples from animals with a large colon impaction were evaluated, there was no evidence of systemic intestinal disease in these donkeys. This evaluation was merely performed for comparative purposes.

As for the horses, there was good consistency (i.e. little variation) in the grades allocated to the donkey tissue sections on the two separate occasions these were evaluated ($P = 0.34$).

Histological examination revealed all samples to be of sufficient quality to be included in the study. Both in the horse and donkey samples, a mild to moderate degree of mucosal

and submucosal lymphocytic infiltration was evident in a number of samples and modest numbers of eosinophils were observed. These inflammatory cells did not appear to extend into the muscularis externa, apart from in the horse large intestinal colic cases where there was a mild perivascular lymphocytic infiltrate and occasional migrating neutrophils in this layer. Although not assessed quantitatively or semiquantitatively, there did not appear to be a difference between diseased and control animals with respect to these mild cellular infiltrates.

Discussion

This study compared the density of PGP 9.5 immunoreactivity in samples from normal and diseased horses and donkeys as part of investigations into the possible causes of intestinal motility disorders in equids. Two previous studies have demonstrated a significant reduction in myenteric neuronal densities in horses with chronic obstructive disorders of the caecum and large colon (Schusser and White, 1997; Schusser *et al.*, 2000). These investigators proposed that the changes observed were linked to the intestinal motility disorders of these animals (Schusser and White, 1997; Schusser *et al.*, 2000). In addition, a reduction in neurons of the enteric nervous system has been described in the dysautonomia of equids, grass sickness (Doxey *et al.*, 1992). These previous studies were carried out using histopathological evaluation of the tissue samples.

Investigators have also evaluated PGP 9.5 as a neuronal marker in human intestinal motility disorders. This protein is involved in the ubiquitin–proteasome system, which is a major pathway for selective protein degradation (Hershko and Ciechanover, 1992), thereby forming an essential part of the normal cellular processes in the cells where it is present. The human disorders where this protein has been used as part of the investigation include conditions such as Hirschprung's disease (Sams *et al.*, 1992; Oh *et al.*, 2002) and idiopathic

megarectum and megacolon (Gattuso *et al.*, 1996, 1997). These studies demonstrated a significant reduction in labelled neurons in the affected areas of intestine as well as documenting that PGP 9.5 provides a reliable and sensitive means of identifying neurons in the intestinal tract (Sams *et al.*, 1992; Gattuso *et al.*, 1996, 1997; Oh *et al.*, 2002). PGP 9.5 has also been shown to be a useful general marker for enteric neurons in recent horse studies, both in health and disease (Milne *et al.*, 2005; Chiocchetti *et al.*, 2009a, b).

The subtle subjective difference observed between donkey and horse PGP 9.5 immunoreactivity was interesting. This may reflect a difference between the two species, but it is important to acknowledge the more geriatric nature of the donkey cases compared with the horse cases, which may or may not have had an effect. This reflects the access to clinical material in these respective caseloads. There has been some research on changes that occur in the ageing intestine, interest possibly stimulated by the increased life expectancy of people and the concomitant medical issues that may emerge. Neuronal loss in the myenteric plexus, as well as neuromuscular dysfunction, has been demonstrated to occur with human ageing (Hall, 2002; Hanani *et al.*, 2004). In a study involving horses up to 20 years of age, Doxey *et al.* (1995) found no decline in the number of small intestinal enteric neurons with increasing age. Additionally, Schusser and White (1997) found no significant difference in colonic neuronal density in normal horses that were 8 years old or less compared with animals over 8 years old. In this present study there was strong immunoreactivity for PGP 9.5 with advancing age in both species. Ideally, future studies should evaluate the enteric nervous system in younger donkeys, but the nature of clinical studies means that it is often only tissue from older donkeys that is available.

In the current study, there was no overall significant reduction in the neuronal densities in the groups of normal versus diseased animals. However, there were two horses with a large colon obstructive disorder (left and right large colon displacement, respectively) that did have

a marked reduction in PGP 9.5 immunoreactivity. Interestingly, one of these horses had a history of previous colic episodes including surgery (for the same lesion, a right large colon displacement) and it seems reasonable to speculate that this could be part of the disease process as proposed by Schusser *et al.* (2000). These two horses also had a significant reduction in ICC density that could also have contributed to the motility problems. In examining the pathophysiology of equine intestinal dysmotility it can be difficult to ascertain whether a finding such as a change in intestinal cellular profile is a primary cause of the syndrome or an effect due to the intestinal disease process itself. If the change observed is primary, it does beg the question as to whether certain animals are predisposed to developing signs of colic and ileus because of this characteristic. As mentioned above, it has been suggested that the reduction in enteric neurons in horses with chronic large intestinal lesions may be a predisposing cause of colic in these animals (Schusser and White, 1997; Schusser *et al.*, 2000). Conversely, in the horse study of Pavone *et al.* (2012), it was suggested that the enteric nervous system and ICC changes observed may have been secondary to the colic episodes. Fintl *et al.* (2004) suggested that the ICC changes in horse large intestinal obstructive disorders may have been a possible cause rather than effect of the colic episodes. Such disparate findings may simply reflect the difficulties of large animal clinical research studies. By their very nature, studies of colic in equids often encompass heterogeneous disorders, making direct comparisons difficult. It is possible that different case cohorts in the various studies may encompass disease processes with differing pathophysiology that may or may not directly damage cellular components such as ICC or neurons. In addition, different studies often evaluate facets of the intestinal motility control system using different methodological approaches, such as alternative antibodies raised against neural elements or different stains, again hindering direct comparison. Furthermore, it should be recognized that colic is a multifactorial syndrome (Archer and Proudman, 2006); intestinal cellular profiles

may have a role, but also factors such as management and dental disease (to name but two) may be important. In the donkeys in the current study there were no animals with a marked reduction in neuronal density in either of the two groups, suggesting that disruptions to the enteric neurons were not involved in the disease process. The poor dental condition of most of the donkeys in the current study has been documented previously (Fintl *et al*., 2010a) and it is plausible that this may have been a contributing factor to the development of colic in the affected animals, rather than disruption to the enteric neurons and ICC which appeared normal.

In conclusion, it appears that the general distribution and density of enteric neurons in the donkey are very similar to that of the horse. PGP 9.5 is a useful general enteric neuronal marker in the horse and donkey and it was also able to reveal strong immunoreactivity in older animals. It was not possible to demonstrate significant changes in enteric neuronal densities in the groups of animals with intestinal disease compared with the control groups, apart from two diseased horses (out of 28 with intestinal disease) where there was a reduction in PGP 9.5 immunoreactivity. Apart from these two animals, this total cohort finding differs from some previously observed findings in horses with intestinal disease and may therefore reflect the different pathophysiological processes occurring in varying intestinal conditions resulting in colic in the donkey and horse.

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Conflict of Interest Statement

All authors declare that there are no conflicts of interest.

References

Archer DC, Proudman CJ (2006) Epidemiological clues to preventing colic. *Veterinary Journal*, **172**, 29–39.

Chiocchetti R, Bombardi C, Mongardi Fantaguzzi C, Russo D, Venturelli E *et al*. (2009a) Intrinsic innervation of the ileocaecal junction in the horse: preliminary study. *Equine Veterinary Journal*, **41**, 759–764.

Chiocchetti R, Bombardi C, Mongardi Fantaguzzi C, Venturelli E, Russo D *et al*. (2009b) Intrinsic innervation of the horse ileum. *Research in Veterinary Science*, **87**, 177–185.

Cox R, Proudman CJ, Trawford AF, Burden F, Pinchbeck GL (2007) Epidemiology of impaction colic in donkeys in the UK. *BMC Veterinary Research*, **3**, 1.

Doxey DL, Pogson DM, Milne EM, Gilmour JS, Chisholm HK (1992) Clinical equine dysautonomia and autonomic neuron damage. *Research in Veterinary Science*, **53**, 106–109.

Doxey DL, Pearson GT, Milne EM, Gilmour JS, Chisholm HK (1995) The equine enteric nervous system – neuron characterization and distribution in adults and juveniles. *Veterinary Research Communications*, **19**, 433–449.

Dukti S, White NA (2009) Prognosticating equine colic. *Veterinary Clinics of North America Equine Practice*, **25**, 217–231.

Du Toit N, Gallagher J, Burden FA, Dixon PM (2008). Post-mortem survey of dental disorders in 349 donkeys from an aged population (2005–2006). Part 2: Epidemiological studies. *Equine Veterinary Journal*, **40**, 209–213.

Fintl C, Hudson NPH, Mayhew IG, Edwards GB, Proudman CJ *et al.* (2004). The interstitial cells of Cajal (ICC) in equine colic: an immunohistochemical study of horses with obstructive disorders of the small and large intestines. *Equine Veterinary Journa*l, **36**, 474–479.

Fintl C, Hudson NPH, Pearson GT, Gallagher J, Mayhew IG (2010a) A study of the interstitial cells of Cajal in aged donkeys with and without intestinal disease. *Journal of Comparative Pathology*, **142**, 242–247.

Fintl C, Pearson GT, Mayhew IG, Lowden CS, Hopwood PA *et al.* (2010b) Comparative analysis of c-kit gene expression and c-Kit immunoreactivity in horses with and without obstructive intestinal disease. *Veterinary Journal*, **186**, 64–69.

Gattuso JM, Hoyle CH, Milner P, Kamm MA, Burnstock G (1996) Enteric innervation in idiopathic megarectum and megacolon. *International Journal of Colorectal Disease*, **11**, 264–271.

Gattuso JM, Kamm MA, Talbot IC (1997) Pathology of idiopathic megarectum and megacolon. *Gut,* **41**, 252–257.

Hall KE (2002) Aging and neuronal control of the GI tract. II. Neural control of the aging gut: can an old dog learn new tricks? *American Journal of Physiology*, **283**, G827–G832.

Hanani M, Fellig Y, Udassin R, Freund HR (2004) Age-related changes in the morphology of the myenteric plexus of the human colon. *Autonomic Neuroscience: Basic and Clinical*, **30**, 71–78.

Hershko A, Ciechanover A (1992) The ubiquitin system for protein degradation. *Annual Review of Biochemistry*, **61**, 761–807.

Hudson NPH, Mayhew IG, Pearson GT (2001). A reduction in interstitial cells of Cajal in horses with equine dysautonomia (grass sickness). *Autonomic Neuroscience: Basic and Clinical*, **92**, 37–44.

Milne EM, Fintl C, Hudson NPH, Pearson GT, Mayhew IG *et al.* (2005) Observations on the interstitial cells of Cajal and neurons in a recovered case of equine dysautonomia (grass sickness). *Journal of Comparative Pathology*, **133**, 33–40.

Oh J-T, Han A, Yang W-I, Han SJ, Choi SH *et al.* (2002) Morphometric evaluation of PGP 9.5 and NCAM expressing nerve fibers in colonic muscle of patients with Hirschprung's Disease. *Yonsei Medical Journal*, **43**, 31–36.

Pavone S, Gialletti R, Pepe M, Onofri A, Mandara MT (2012) Histological and immunohistochemical studies of changes in myenteric plexuses and in interstitial cells of Cajal associated with equine colic. *Research in Veterinary Science*, **93**, 350–359.

Prince D, Corcoran BM, Mayhew IG (2003) Changes in nasal mucosal innervation in horses with grass sickness. *Equine Veterinary Journal*, **35**, 60–66.

Sams VR, Bobrow LG, Happerfield L, Keeling J (1992) Evaluation of PGP 9.5 in the diagnosis of Hirschprung's Disease. *Journal of Pathology*, **168**, 55–58.

Schusser GF, White NA (1997) Morphologic and quantitative evaluation of the myenteric plexuses and neurons in the large colon of horses. *Journal of the American Veterinary Medical Association*, **210**, 928–934.

Schusser GF, Scheidemann W, Huskamp B (2000) Muscle thickness and neuron density in the caecum of horses with chronic recurrent caecal impaction. *Equine Veterinary Journal*, **Suppl. 32**, 69–73.

Tinker MK, White NA, Lessard P, Thatcher CD, Pelzer KD *et al.* (1997) A prospective study of equine colic incidence and mortality. *Equine Veterinary Journal*, **29**, 448–453.

Traub-Dargatz JL, Kopral CA, Seitzinger AH, Garber LP, Forde K *et al*. (2001) Estimate of the national incidence of and operation-level risk factors for colic among horses in the United States, spring 1998 to spring 1999. *Journal of American Veterinary Medical Association*, **219**, 67–71.

White NA (1990) Epidemiology and etiology of colic. In: *The Equine Acute Abdomen*. NA White, Ed., Lea & Febiger, Philadelphia, pp. 49-64.

Figure Legends

Fig. 1. PGP 9.5 immunoreactivity in the mucosa and submucosa of the ileum of a control horse. Immunoreactivity is present in nerve fibres (arrows) and a submucosal ganglion (asterisk). Nerve fibres can be seen in networks (arrows) extending into the mucosal villi.

Fig. 2. PGP 9.5 immunoreactivity in the pelvic flexure of a control horse. Arrows indicate labelled nerve fibres. LM, longitudinal muscle layer; CM, circular muscle layer; MG, myenteric ganglion.

Fig. 3. PGP 9.5 immunoreactivity in the pelvic flexure of a horse with an obstructive large colon disorder. Arrows indicate labelled nerve fibres. LM, longitudinal muscle layer; CM, circular muscle layer; MG, myenteric ganglion.

Fig. 4. PGP 9.5-immunoreactive nerve fibres and ganglia (asterisks) in the myenteric plexus region of the ileum of a control donkey. LM, longitudinal muscle layer; CM, circular muscle layer.

Fig. 5. PGP 9.5 immunoreactivity in the mucosa and submucosa of the ileum of a control donkey. Immunoreactivity is present in nerve fibres (arrows) and a submucosal ganglion (asterisk). Nerve fibres can be seen in networks (arrows) extending into the mucosal villi.

Table 1

Details of horses in the study

Animal	*Age (years)*	*Sex*	*Clinical diagnosis*	*Sample collected*	*Previous colic episodes*
Controls					
1	4	MN	Lameness	Il/ PF	No
2	11	MN	Lameness	Il/ PF	No
3	1	M	Lameness	Il/ PF	No
4	12	MN	Lameness	Il	No
5	16	F	Lameness	Il	No
6	11	MN	Lameness	Il	No
7	33	MN	Lameness	Il	No
l 8	18	F	Reproductive problems	Il	No
9	12	F	Lameness	Il	No
10	6	MN	Lameness	Il	No
11	7	MN	Lameness	Il	No
12	31	MN	Cardiac dysfunction	Il	No
13	19	MN	Lameness	PF	No
14	3	MN	Lameness	PF	No
15	15	F	Lameness	PF	No
16	9	MN	Lameness	PF	No
17	2	F	Neurological problem	PF	No
SI Colic					
1	7	MN	EFE	Il	No
2	12	MN	EFE	Il	No
3	24	F	Lipoma	Jej	No
4	14	MN	Meckel's diverticulum	Jej	No
5	8	F	Mesenteric rent	Jej	No
6	12	MN	Eosinophilic	Il	No
7	19	MN	Lipoma	Il	No
8	14	MN	EFE	Il	No
9	24	F	Lipoma	Jej	No
LI Colic					
10	6	MN	RLCD	PF	Yes + Surgery
11	5	MN	RLCD	PF	Yes + Surgery
12	3	F	PFI (SI)	PF	Yes + Surgery
13	24	MN	PFI (SI)	PF	Yes
14	11	F	PFI (SI)	PF	Yes
15	14	F	LCT	PF	No
16	11	F	LCT	PF	No
17	17	F	LCT	PF	No
18	12	MN	LCT	PF	No
19	4	MN	RLCD	PF	No
20	14	F	LCT	PF	Yes + Surgery
21	5	MN	LLCD	PF	No
22	11	F	RLCD	PF	No
23	11	MN	RLCD	PF	No
24	5	F	LLCD	PF	Yes
25	9	F	LLCD	PF	No
26	9	MN	LCT	PF	No
27	2	F	LLCD	PF	No
28	3	F	LLCD	PF	No

SI, small intestine; LI, large intestine; F, female; M, male; MN, male neutered; Il, ileum; Jej, jejunum: PF, pelvic flexure; EFE, epiploic foramen entrapment; Eosinophilic, focal strangulating eosinophilic lesion; PFI (SI), pelvic flexure impaction (sand impaction); LCT, large colon torsion; LLCD, left large colon displacement; RLCD, right large colon displacement

Table 2

Details of donkeys in the study

	Animal	*Age (years)*	*Sex*	*Reason for humane destruction*	*Final diagnosis*
Control	1	32	F	Lame	Laminitis
group	2	26	MN	Neurological signs	Unknown
	3	31	MN	Dental	Dental
	4	28	F	Renal failure	Nephrosis
	5	26	F	Respiratory signs	Pneumonia
	6	26	F	Colic	CLF
Colic group	7	27	MN	Weight loss	PFI
	8	35	MN	Colic	PFI
	9	33	M	Suspected liver disease	PFI
	10	32	MN	Weight loss	PFI
	11	28	MN	Distended abdomen	PFI
	12	27	MN	Distended abdomen	PFI

F, female; M, male; MN, male neutered; PFI, pelvic flexure impaction; LI colic, large intestinal colic; CLF, chronic liver fibrosis

Table 3

***P*-values for comparison of PGP 9.5 density grades in normal and diseased horses**

	Normal versus diseased small intestine lesion	*Normal versus diseased large intestine lesion*
Mucosa	0.63	0.11
Circular muscle layer	0.34	0.19
Myenteric plexus	0.84	0.97

Table 4

***P*-values for comparison of PGP 9.5 density grades in normal and diseased donkeys**

	Normal versus small intestine from animals with large intestinal disease	*Normal versus diseased large intestine*
Mucosa	0.06	0.64
Circular muscle layer	0.38	0.17
Myenteric plexus	0.19	0.34

Figures

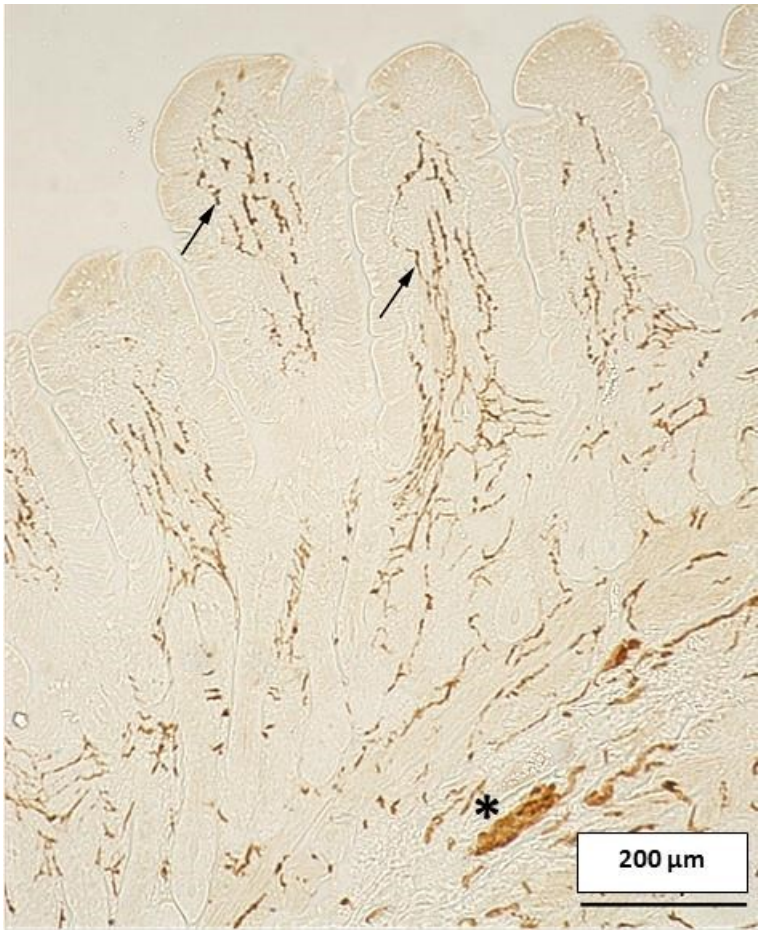

Fig. 1: PGP 9.5 immunoreactivity in the mucosa and submucosa of the ileum of a control horse. Immunoreactivity is present in nerve fibres (arrows) and a submucosal ganglion (asterisk). Nerve fibres can be seen in networks (arrows) extending into the mucosal villi.

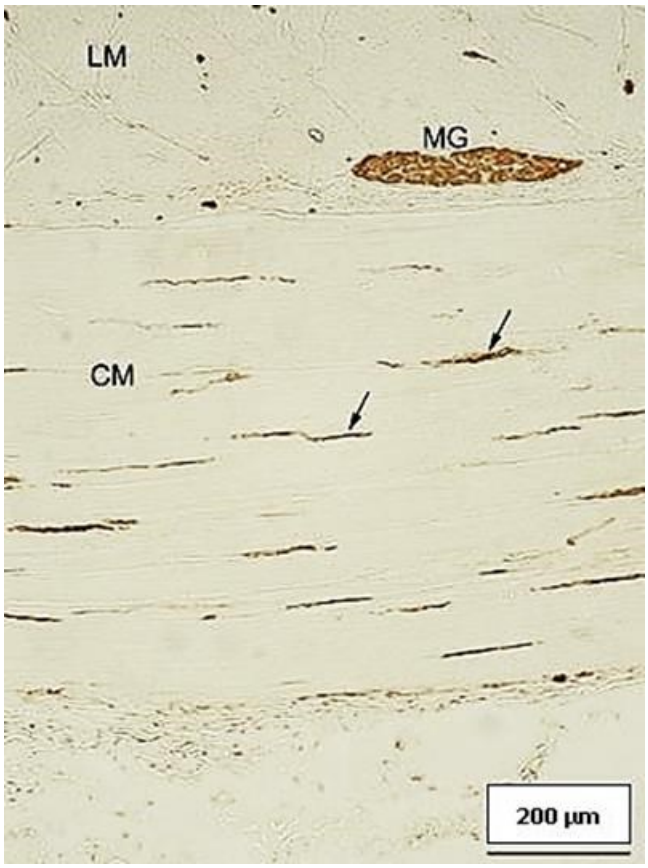

Fig. 2: PGP 9.5 immunoreactivity in the pelvic flexure of a control horse. Arrows indicate labelled nerve fibres. LM: longitudinal muscle layer; CM: circular muscle layer; MG: myenteric ganglion.

Fig. 3: PGP 9.5 immunoreactivity in the pelvic flexure of a horse with an obstructive large colon disorder. Arrows indicate labelled nerve fibres. LM: longitudinal muscle layer; CM: circular muscle layer; MG: myenteric ganglion.

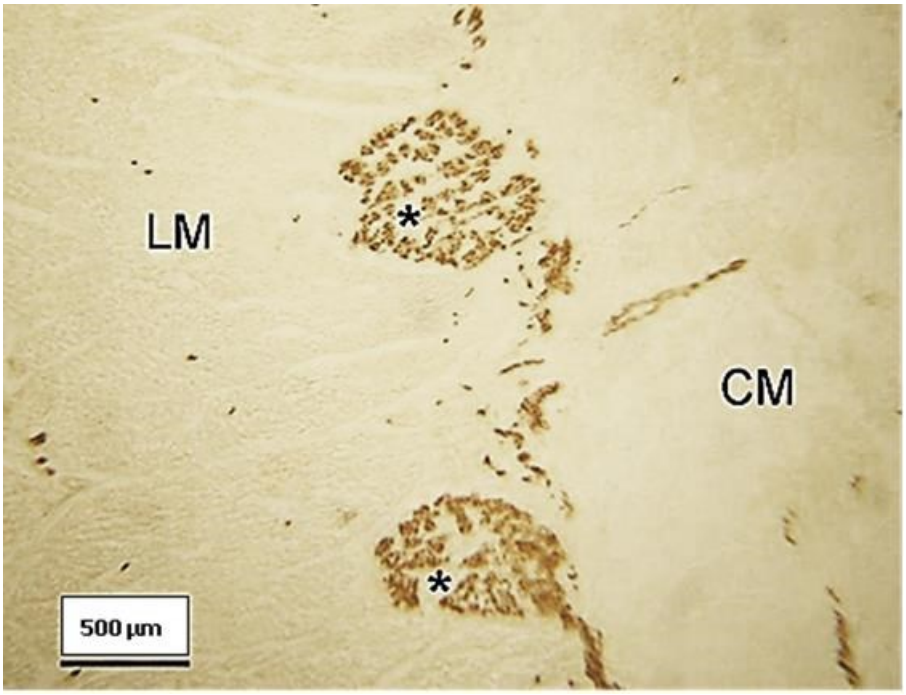

Fig. 4: PGP 9.5-immunoreactive nerve fibres and ganglia (asterisks) in the myenteric plexus region of the ileum of a control donkey. LM: longitudinal muscle layer; CM: circular muscle layer.

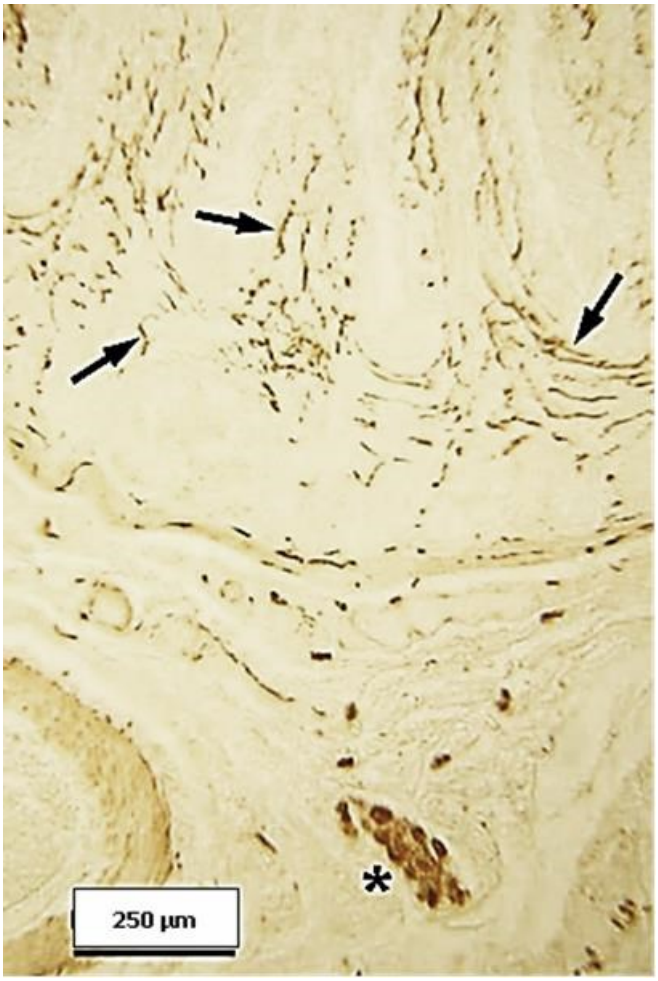

Fig. 5: PGP 9.5 immunoreactivity in the mucosa and submucosa of the ileum of a control donkey. Immunoreactivity is present in nerve fibres (arrows) and a submucosal ganglion (asterisk). Nerve fibres can be seen in networks (arrows) extending into the mucosal villi.